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This project achieved all of its goals. Preparation of prostate tissue slices was optimized by a variety of technological improvements. Optimal culture conditions were identified for the maintenance of structure and function of cultured tissue slices for at least 48 hours. The requirement for androgen to maintain the secretory epithelium in cultured tissue slices was demonstrated. The effects of a number of clinically relevant compounds on gene expression, protein expression, structure and function were effectively analyzed in tissue slice cultures. We conclude that challenges still remain to achieve long-term culture of tissue slices with full preservation of structure and function, but unique preclinical information can already be gained from this methodology as it has been devbeloped so far.

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INTRODUCTION

Our project was designed to address the problem that no experimental model system accurately predicts the clinical efficacy of a drug against prostate cancer. Monolayer cell cultures, co-cultures combining stromal and epithelial cells, three-dimensional cultures in matrix supports, and xenografts are currently used to evaluate pre-clinical activity of experimental compounds. Numerous agents that kill or inhibit the growth of prostate cancer cells in one or more of these model systems have been identified, yet an effective chemotherapeutic agent for prostate cancer is still not available. A more realistic model of prostate cancer is needed, and we suggested that tissue slice cultures could fill this need.

Tissue slice cultures have been used for biochemical studies for several decades. Although typically viable for only a short period of time, tissue slice cultures from liver, kidney, heart and lung were nevertheless used for many diverse studies, including toxicology, pharmacology, organ preservation and metabolism. In the early 1990's, methodology was developed for long term maintenance of tissue slice cultures. However, the recent advancement that promised to extend the use of tissue slice cultures to a wide variety of organs and applications was precision cutting. This is accomplished with an automated microtome (the Krumdieck tissue slicer, for example), which permits the rapid preparation of aseptic slices of live tissues with nearly identical dimensions. This instrument eliminated the major source of error in tissue slice work, namely, the use of slices of different and uneven thickness with irregular, non-reproducible damage at the cut surfaces. This improvement eliminated the variable of gas and nutrient exchange by diffusion that exists among unequally or unevenly cut slices.

Prior to receiving our research award from DAMD, we purchased the Krumdieck tissue slicer and performed pilot studies to begin to master the technical challenges of prostate tissue slice culture. We used a special core borer to obtain tissue from a radical prostatectomy specimen. This core of fresh tissue was mounted on the Krumdieck microtome and thin sections were cut by a rapidly reciprocating, disposable microtome blade. Throughout the cutting procedure, the tissue remains immersed in chilled physiologic fluid. Upon cutting, the thin sections gently shoot into a reservoir of physiologic fluid, and are then transferred to grids in culture vessels with the aid of a tool that resembles a miniaturized minnow scoop. The tissue slices are rotated in a specially designed apparatus that eliminates conventional mechanical shaking, which inevitably leads to disintegration of tissue at the slice-medium interface. In our pilot study, we were able to obtain cores of fresh tissue, cut thin sections and successfully transfer the sections to culture vessels. An additional challenge was to recover the thin sections for analysis after culture. We were able to fix the thin sections, transfer them to embedding cassettes, dehydrate and embed in paraffin, and cut 5 μm sections for histological analysis after staining with Hematoxylin and Eosin (H & E). We also performed immunohistochemical analyses to check maintenance of function.

Our proposed plan was to improve our technical abilities to prepare, process and evaluate tissue slice cultures and to develop optimal culture conditions for these tissues *in vitro*. Our long term objective was to use tissue slice cultures as a pre-clinical model system to screen therapeutic agents. In this Final Report, we summarize our studies and results.

BODY

Our **first designated task was to optimize preparation of prostatic tissue slices**. In year 1 of this project, we purchased an automated tissue coring press to replace our hand-held, manual tissue coring tool. The use of the automated press enabled us to obtain uniform tissue cores of consistent and uniform diameter from surgical specimens. While the automated corer was an improvement, we still had difficulty in obtaining optimal cores from every surgical specimen. We thought that this was due to the particular constituency of prostate tissue, and to the variability and heterogeneity of constituency among specimens, presumably related to histological make-up of the specimens. For example, some tissues are quite soft while others are fibrous. One unexpected problem was that the corer became dull after only a few uses, which we thought was due to fibrous tissue. This necessitated precision sharpening at the manufacturer in Alabama, which caused additional expense and delays in year one. In year 2, another part on the microtome required replacing. However, subsequently all equipment remained in good repair and we had no additional problems after the set backs of the initial years.

In addition to purchase of the automated coring press, another improvement was made by boring longer tissue cores. The cores are embedded in agarose prior to placing in the tissue holder on the microtome, and the cores had been detaching from the tissue holder after only about half of the core had been sliced. We determined that boring longer cores from the prostate specimen decreased this problem. We also did a study to determine how thin we could realistically cut tissue slices. Although we could slice sections as thin as 90 μm, put them into culture, retrieve the sections after culture, fix and embed in paraffin and cut 5 μm sections for histologic analysis (shown in figures of the annual progress report in year 2), we found that sections cut in the range of ~ 200 - 300 μm were easier to work with and yielded more material for analysis. Therefore, for the remainder of our studies, we routinely sliced sections at ~ 300 μm.

Another goal of task 1 was to optimize the fixation and embedding protocol for tissue slice cultures. One problem that we encountered was the loss of a fair amount of the tissue slice as we trimmed the face of the embedded block. Our idea to prevent this is was to lay the tissue slice on a piece of nitrocellulose filter paper as it was embedded in paraffin in the cassette. The filter paper could then serve as a "marker" on which to trim the block, thereby preserving the limited amount of tissue in each block. While this was good in theory, in practice it didn't work. Presumably because the filter paper is harder than the tissue, once the microtome started cutting the filter paper, the small tissue slice was pulled out of the block.

Therefore, we tested another idea, which was to paint the face of the fixed tissue slice with India ink. This helped with alignment of the block, although the ink tended to seep through the ducts of the tissue and somewhat obscured histologic and immunohistochemical analyses. In the end, we usually did not paint the block, but instead cut sections through the block for storage once we aligned the block, so additional tissue was not wasted by having to re-align the block at a later date.

Determining optimal fixation methods was another element of Task 1. The goal was to achieve good histological preservation without having to use Antigen Retrieval methods for immunohistochemical staining. Formalin achieves the former but many antibodies require the use of retrieval after formalin fixation. Therefore we conducted several studies testing a variety of fixatives, including formalin, Histochoice, 2% paraformaldehyde, and "Hope" fixative. Cultured or uncultured tissue slices were placed in the fixatives for periods ranging from 30 minutes to 2 hours to overnight. The slices were then embedded in paraffin and sectioned at 5

μm. The tissue sections were then stained with hematoxylin and/or analyzed by immunohistochemistry.

Histological preservation was good with paraformaldehyde and formalin and adequate with Histochoice (shown in figures in previous Annual reports). Histologic preservation with HOPE fixative, however, was horrible. This fixative, made by a company in Germany, is touted to preserve antigenicity and even enzymatic activity. After we saw the list of reagents, the reason for this seemed apparent, since the HOPE solution is mainly a mixture of sugars and amino acids. An incubation in ethanol does not occur until after an overnight incubation in HOPE solution, and it seems that our tissue slices disintegrated during this time. Immunohistochemical staining with antibody against keratin 18, which usually doesn't require Antigen Retrieval, was good with all of the fixatives (shown in previous Annual Reports). The only fixative which did not require antigen retrieval to reveal staining of the basal cell keratins, 5 and 14, was Histochoice. In the end, we decided that overnight fixation in Histochoice gave the best balance between histologic preservation and preservation of antigenicity.

Given the technical challenges with fixing, sectioning and staining the tissue slices, we investigated a faster way to evaluate tissue slices. We performed immunoblot analysis to follow protein expression in tissue slices with time in culture and +/- various treatments. We reasoned that adjacent tissue slices are fairly similar in histologic makeup. Therefore, if we cut one slice as an uncultured control, and the adjacent slice was cultured, we could make protein lysates from each and monitor changes in protein expression in the cultured slices. In year 2, we evaluated our ability to make protein lysates from tissue slices. We purchased a Dounce homogenizer and prepared protein lysates from tissue slices at t_0 (uncultured), and at 6 and 24 hrs after culture. Approximately 300 μg of protein were obtained from each tissue slice, and 20 μg of each sample were run on an SDS-polyacrylamide gel and transferred to a blot. Labeling with actin showed ~ equivalent levels of actin in each sample (shown in last year's annual report), providing us with an appropriate "housekeeping" protein to compare other proteins. In year 3, we continued to explore this technique as a rapid method to monitor the phenotype of tissue slice cultures, with confirmation by immunohistochemistry of fixed specimens of interesting results. Results from this approach are described in subsequent sections of this report.

The **second task was to develop optimal culture conditions for the maintenance of tissue structure and function.** In the first year, we determined that "Complete PFMR-4A", consisting of defined basal media supplemented with cholera toxin, epidermal growth factor, insulin, phosphoethanolamine, hydrocortisone, selenium, alpha-tocopherol, retinoic acid, bovine pituitary extract, and gentamicin (Peehl, 1992), maintained fairly good histological and functional preservation of tissue slices cultured up to 24 hours. Basal epithelial cells, secretory epithelial cells and stromal smooth muscle remained intact, as demonstrated by immunohistochemical staining with appropriate markers. We found that the degeneration of secretory cells that began to appear by 48 hours could be lessened if androgen (10 nM R1881) was included, so this formulation was routinely used for the remainder of our studies. These experiments addressed **Task 3, which was to test the validity of tissue slice cultures as an accurate in vitro model by depriving the tissue slices of androgen.** While our initial studies suggested that secretory cells were maintained longer in the presence of androgen, we confirmed this with additional immunohistochemical studies.

We performed several studies to address **Task 4, to test agents used clinically for their effects on tissue slice cultures.** Docetaxel is one of the leading chemotherapeutic agents used to

treat prostate cancer, so we treated tissue slice cultures +/- 1 μM docetaxel for 48 hours. Histologic evaluation suggested an increased level of apoptosis in tissue sections treated with docetaxel compared to untreated tissue slices (shown in year 2 annual report). This was confirmed by immunohistochemical labeling with antibody against cleaved caspase-3, a marker of apoptosis. We also tested doxazosin, an alpha-adrenoceptor antagonist that is used to treat benign prostatic hyperplasia. In monolayer cultures, doxazosin induces apoptosis in prostatic epithelial cells. Histological observations of tissue slices treated for 48 hr +/- 50 μM doxazosin suggested that there was more cellular degeneration in the treated than in the untreated tissue culture slices (shown in year 2 progress report).

In year 3, we evaluated the effects of several clinically relevant compounds on tissue slice cultures. One of these compounds was triptolide, a diterpene triepoxide purified from the herb Trypterygium wilfordii hook F. Triptolide is in phase 1 clinical trials to treat cancer. We previously described the anti-proliferative and pro-apoptotic effects of triptolide on primary cultures of human prostatic epithelial cells (Kiviharju et al., 2002). Those pre-clinical studies suggested that triptolide might be an effective chemotherapeutic agent against prostate cancer. We therefore sought to gain additional supportive pre-clinical results with tissue slice cultures. Our goals were to determine whether triptolide upregulated p53 and induced apoptosis in epithelial cells in tissue slice cultures as it did in primary cultures of epithelial cells. Figures 1 and 2 show that this indeed was the case. In Figure 1, apoptotic cells are seen in the epithelium of tissue slice cultures treated for 24 hours with triptolide but not in untreated slices. After 48 hours of exposure to triptolide, the epithelium was severely degenerated. Figure 2 shows the increase in p53 protein that occurred in the epithelium of tissue slice cultures treated with triptolide. Figure 3 shows the results from immunoblot analysis of proteins extracted from tissue slice cultures. After 4 hours of culture in medium +/1 triptolide, intact keratin 18 is present but cleaved fragments of keratin 18 are not. After 8 hours of culture, a cleavage fragment of keratin 18 is visible in the protein isolated from tissue cultured with triptolide but not in the protein from untreated tissue. This result corresponds with the induction of apoptosis by triptolide shown in Figure 1 by histological analysis, because cleavage of keratin 18 is associated with apoptosis ( ). Keratin 14, a marker of the basal epithelium, is also shown in Figure 3. The levels of keratin 14 were similar in tissue slices cultured with or without triptolide, suggesting that the effects of triptolide mainly occur in the secretory rather than the basal epithelium. Figure 4 shows the cleavage fragment of p53 that occurred in triptolide-treated cultures. Cleaved p53 has also been associated with apoptosis.

Another clinically relevant agent that we tested was γ-irradiation. Radiotherapy is commonly used to treat prostate cancer, yet the biology of radiation response of prostate cells is not well-defined. We previously made the observation that primary cultures of prostatic epithelial cells do not upregulate p53 in response to irradiation, nor do they undergo cell cycle arrest or apoptosis (Girinsky et al., 1995). This response is different from established cell lines or prostatic stromal cells, and raises important questions regarding mechanisms of radiotherapy in prostate cancer. It is critical to demonstrate that this result is not some sort of artifact associated with cell culture, but it is difficult to retrieve prostate tissues following radiotherapy from patients to see if this happens in vivo. Therefore, we evaluated irradiated tissue slice cultures. By immunohistochemistry, we noted a robust upregulation of p53 protein in secretory epithelial cells and stroma in irradiated tissue slices (Figure 5). In contrast, p53 was not upregulated in the majority of basal epithelial cells. Since the phenotype of primary cultures of prostatic epithelial cells is most similar to that of basal cells, our results from tissue slice cultures

supplement our previous work and strengthen our hypothesis that basal (progenitor) cells protect themselves from loss through apoptosis by blocking induction of p53 in response to DNA-damaging agents. The information that we uniquely gained from the tissue slice cultures makes us confident that our results from primary cultures were not a cell culture artifact but indeed are representative of real prostate biology.

Similarly, we also initiated studies with tissue slice cultures to expand our pre-clinical work with vitamin D. Studies from our laboratory have contributed to the large volume of research that has led to the application of vitamin D as a preventive and therapeutic agent against prostate cancer. Recently, we performed microarray analysis of vitamin D-treated primary cultures to identify molecular targets of vitamin D in prostatic epithelial cells (Peehl et al., 2004). The gene encoding vitamin D 24-hydroxylase was the most highly upregulated by vitamin D in cultured prostatic epithelial cells. Tissue slice cultures were treated with and without 50 nM of vitamin D for 6 hours, RNA was isolated, and levels of 24-hydroxylase RNA were measured by real-time polymerase chain reaction (PCR). This was the first time that we isolated RNA from tissue slice cultures, and we determined that the RNA was of good quality and in large enough quantity for multiple PCR analyses. Furthermore, we observed a large increase in the level of 24-hydroxylase RNA in the vitamin D-treated tissue slice culture (Figure 6), as anticipated from the cell culture results. This experiment sets the stage for analysis of other genes implicated as vitamin D targets from our cell culture studies. As for the previously described radiation studies, this experiment illustrates the potential value of tissue slice cultures – it is difficult to obtain tissue from the prostates of men treated with vitamin D, so tissue slice cultures can be used as a surrogate model.

KEY RESEARCH ACCOMPLISHMENTS

- Optimized preparation of prostatic tissue slices (task 1) by using an automated core borer, obtaining longer cores, cutting sections of ~ 200-300 μm, using Histochoice fixative and inking face of block
- Developed optimal culture conditions for the maintenance of tissue structure and function (task 2) by using serum-free medium PFMR-4A supplemented with 10 nM R1881, evaluating structure by histological analysis of hematoxylin-stained fixed sections, and evaluating gene and protein expression of basal epithelium, secretory epithelium and stroma by immunohistochemistry, immunoblot analysis, and real-time PCR
- Tested the validity of tissue slice cultures as an accurate in vitro model (task 3) by showing that the secretory epithelium degenerated in the absence of R1881
- Initiated pilot studies to use tissue slice cultures to screen candidate therapeutic agents for prostate cancer (Task 4) by testing clinically relevant agents including docetaxel, doxazosin, triptolide, vitamin D and irradiation

REPORTABLE OUTCOMES

None.

CONCLUSIONS

While we met the goals of each of our proposed tasks, we conclude that prostate tissue slice culture is a challenging technique. The nature and biology of prostate tissue seemingly present greater difficulties compared to other types of tissues. Foremost among these is the apparent lability of the differentiated secretory epithelium of the prostate. As has been noted in attempts to isolate secretory epithelial cells and maintain them in monolayer culture, the secretory cells in tissue slice cultures quickly lose expression of androgen receptor and prostate-specific antigen and degenerate. This occurred as early as 48 hours after initiation of tissue slice cultures, and we could identify no modification of culture conditions to prevent this. This inability to maintain the differentiated epithelium remains the main obstacle to long-term maintenance of prostate tissue slice culture. Nevertheless, we demonstrated that useful pre-clinical information could be obtained in short-term tissue slice cultures of 48 hours or less. We demonstrated induction of apoptosis by docetaxel and doxazosin in tissue slice cultures, as has been shown in the prostates of men treated with these drugs. This result suggests that tissue slice cultures indeed are a realistic model of the prostate. Similarly, we showed the upregulation of p53 and induction of apoptosis by triptolide, an experimental drug in early clinical trials, in tissue slice cultures. We previously reported these activities of triptolide in primary cultures of prostatic epithelial cells, so these results provide additional evidence that triptolide indeed could be an effective therapeutic agent against prostate cancer. We were also able to use tissue slice cultures to obtain additional evidence about the cell-specific nature of upregulation of p53 by irradiation of tissue slice cultures, which compliments our work with primary cultures and is relevant to radiotherapy of prostate cancer. Although prostate tissue slice culture requires further optimization before it can be widely used, our results suggest that this methodology can be used in a selective manner to provide clinically relevant information not available from any other model system.

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APPENDICES

Figures 1-6.

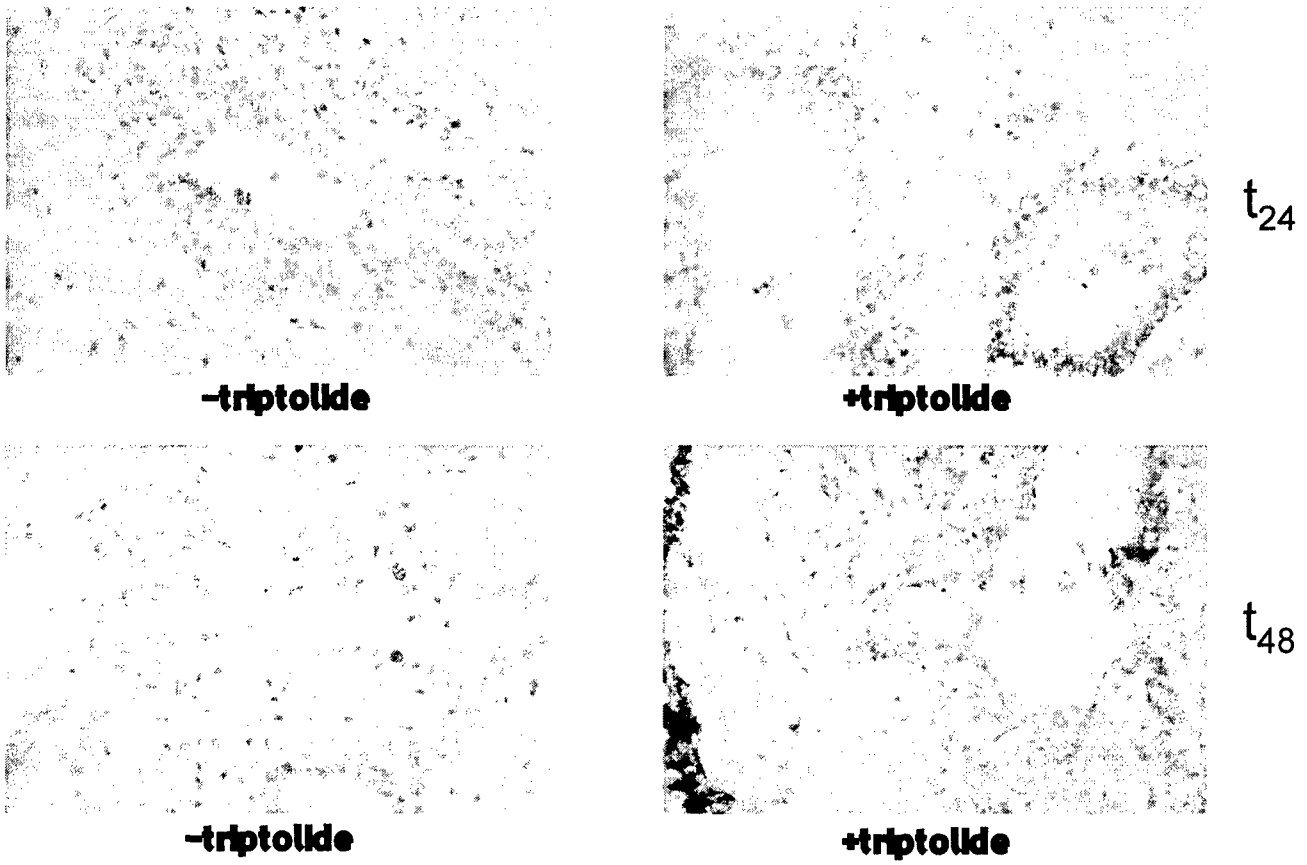

Figure 1. Apoptosis in tissue slice cultures in response to triptolide. Tissue slice cultures were treated +/- triptolide (50 nM) for 24 or 48 hours. By 24 hours, apoptotic cells were clearly visible in the epithelium of treated but not untreated tissues. By 48 hours, the epithelium in treated tissues was degenerated, while remaining intact in untreated tissues.

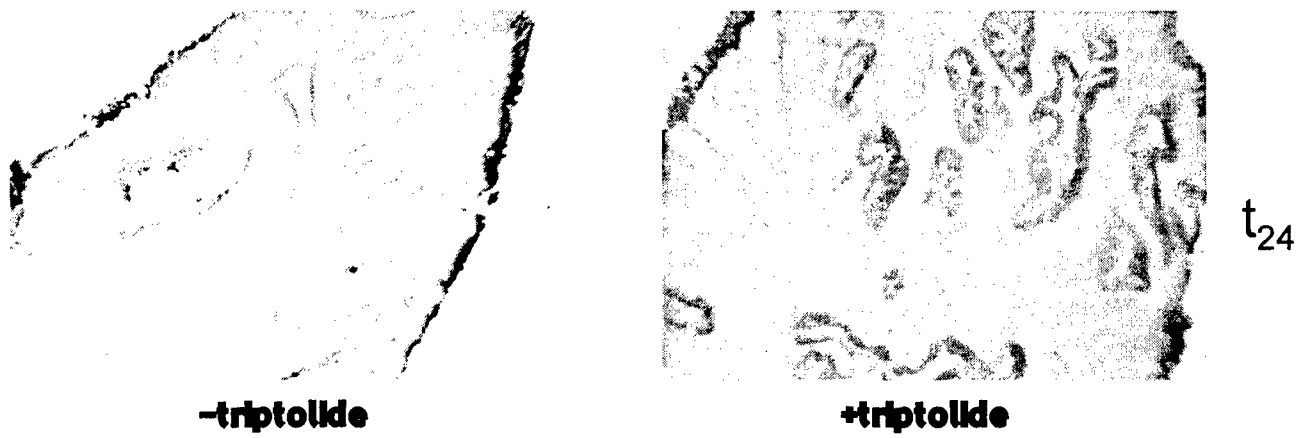

Figure 2. Induction of p53 by triptolide in tissue slice cultures. Tissues cultured +/- triptolide for 8 hours were fixed, embedded and sectioned. The expression of p53 was evaluated by immunochemistry. The p53 protein was dramatically upregulated in the epithelium of p53-treated tissues while remaining low or absent in untreated tissues

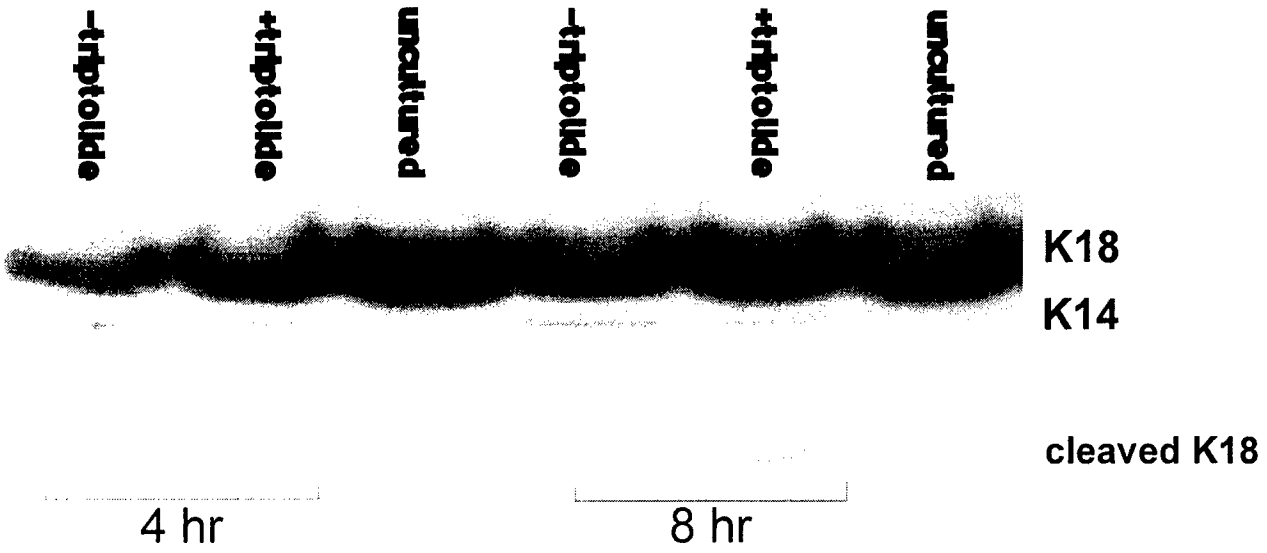

Figure 3. Cleavage of keratin 18 in triptolide-treated tissue slice cultures. Protein lysates from tissue slices incubated for 4 or 8 hours +/- triptolide were analyzed in immunoblots. The intact form of keratin 18 (upper band) was ~equivalent in uncultured tissues and tissues grown +/- triptolide. Keratin 14 (fainter middle band) was increased in tissue slice cultures compared to uncultured tissue, but was equivalent in tissues cultured with or without triptolide. However, a cleavage fragment of keratin 18 (lower band) appeared only in triptolide-treated cultures at 8 hours

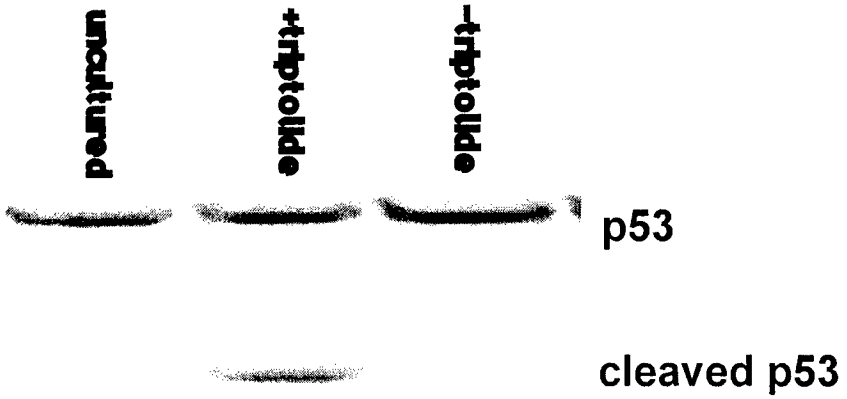

Figure 4. Cleavage of p53 in triptolide-treated tissue slice cultures. Protein lysates from tissue slices cultured +/- triptolide were isolated at 8 hours and analyzed in immunoblots. Intact p53 protein was ~ equivalent in uncultured tissue and tissues cultured +/- triptolide (upper band), but a cleavage fragment (lower band) occurred ony in triptolide-treated cultures.

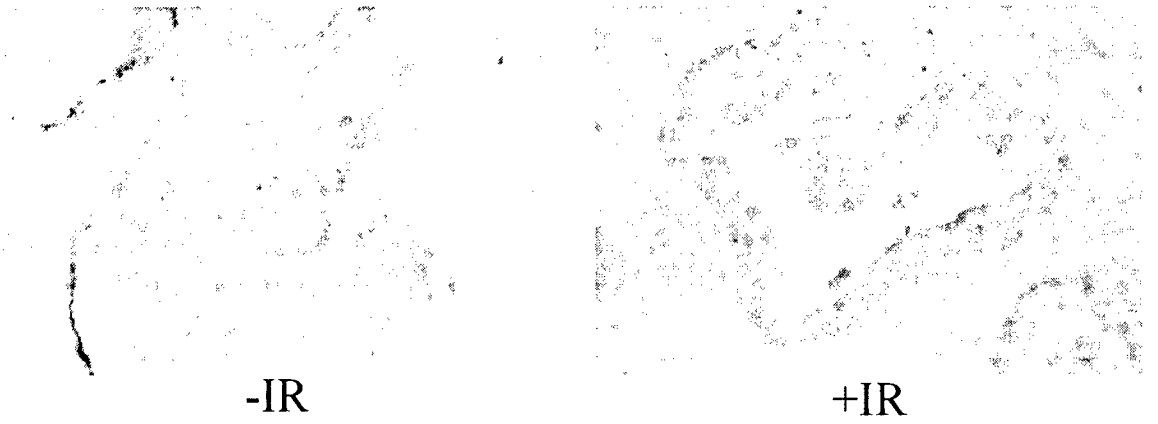

Figure 5. Induction of p53 and cellular localization in irradiated tissue slice cultures. Tissue slice cultures were exposed to 10 Gy of gamma-irradiation. After 24 hours of culture, control (unirradiated) and irradiated tissues were fixed, embedded and sectioned. Immunocytochemical labeling revealed robust induction of p53 more or less uniformly in the stroma of irradiated tissue. The p53 protein was also induced in the epithelium. However, while the majority of secretory epithelial cells were labeled with antibody against p53, basal epithelial cells were rarely labeled.

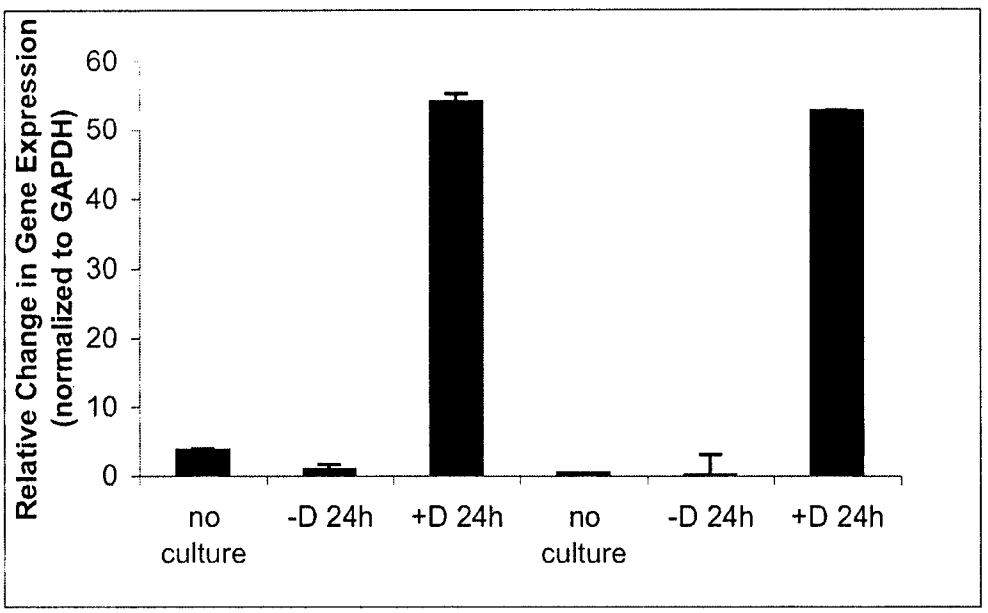

Figure 6. Induction of 24-hydroxylase RNA in tissue slice cultures by vitamin D. Tissue slice cultures were grown +/- vitamin D (50 nM). At 24 hours, RNA was extracted from each tissue slice and processed for analysis. Real-time PCR measurements showed a large induction of 24-hydroxylase RNA by vitamin D, with low basal levels in uncultured tissues and tissues cultured without vitamin D.